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이학석사 학위논문

황소개구리의 혈연인식

Kin recognition in *Rana catesbeiana*

2018 년 8 월

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권 한 솔

황소개구리의 혈연인식
Kin recognition in *Rana*
catesbeiana

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Kin recognition in *Rana catesbeiana*

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Abstract

To test the notion that genetic relatedness and familiarity can influence kin recognition in *Rana catesbeiana* tadpoles - that do not show schooling tendencies - the frequencies in which they contact one another was counted. Genetic relatedness and familiarity were manipulated by assigning *Rana catesbeiana* egg mass prior to hatching to either a common tank to be reared together or separate tanks to be reared separately. Each test had a total of eight tadpoles placed in the 10 cm diameter glass bowl. Four tadpoles were dyed with neutral red and the other four tadpoles were dyed with methylene blue; the different coloration indicated sibship. There were three groups tested; control group was composed of all familiar eight tadpoles of the same sibship dyed differently, mixed group was composed of tadpoles of the different sibship that were dyed differently but were familiar and pure group was composed of tadpoles of the different sibship that was indicated by their different coloration and unfamiliar tadpoles on the same glass bowl. The results indicated that there was no difference to the two variables, genetic relatedness and familiarity, to tadpoles making contact to each other. The tadpoles showed relatively elevated levels of contact to unfamiliar tadpoles to familiar tadpoles which indicated acclimation but also assessment. It is necessary for further study to take place.

Keyword: Kin recognition, bullfrog, social interaction, genetic relatedness, familiarity, *Rana catesbeiana*

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1. Introduction

1.1. Kin Recognition in Amphibians

The ability of an organism to recognize kin has been studied progressively over the past few decades. The degrees of kin recognition vary throughout the animal kingdom, from single-celled organism to man (Hepper 2005). The underlying mechanisms that may lead to organisms to recognize kinship in behavior and the importance of kinship has been long researched. While many view kin recognition to be important, including avoiding incest in mate choice and effective parental care in birds, the mechanism in which this is achieved has been poorly understood in many groups (Hepper 2005). In this study we aimed to investigate tadpole behavior in American bullfrogs that were grouped separately, according to genetic-relatedness and familiarity, that may be important to mechanisms of kin-recognition. Tadpoles of *Rana catesbeiana* manifest occasional social and spherical aggregations in nature (de Avelar et al. 2008) and this laboratory study will assist us in developing theories relevant to their interactions in the wild.

Importance of kin recognition and the influence of kinship to drive animals to respond deferentially has been researched in the past and the impetus was from Hamilton and his research of altruism (Hamilton et al. 1972). This research was later acknowledged by Wilson (1975). Kin recognition was researched in mammals in areas such as parental care and sibling-recognition in birds (Lukas 2013) social structure and gene flow dynamics in chimpanzees (Morin et al. 1994) and in aquatic animals as such as shoaling in zebrafish (Miller et al. 2012). Larval amphibians have

proven model organisms for the field studies for the ease in tracing their community structure and population (Kats et al. 1988, Kiesecker et al. 1999). Kin recognition studies in amphibians are often researched because their kin-recognition modalities are highly tractable (Waldman 1991) and their varied response depending on their genetic relatedness can be efficiently analysed; whereas studies using mammalian study organisms are often cumbersome to be maintained under laboratory settings (Waldman 1991). Recently there has been extensive development of analysis programs and tools available and so, the analysis of behavior has become more accurate and statistically significant (Hepper 2005).

The use of tadpoles in kin recognition studies is popular because recognition of nearby tadpoles is the method by which those aquatic organisms formed schools to deter from predators, increase feeding efficiency and thermoregulation (Adler et al. 1979). The mechanisms as to how organisms achieve kin recognition was lacking significant study (Hepper 2005).

1.2. Conservation Science and American Bullfrogs

Conservation science in the field of environmental science is an interdisciplinary study to protect and care of the natural environment. As many governments believe that economic prosperity is based on continued growth, their active policies place economy on top of conservation priorities (Vitt et al. 2013). The underlying human greed has reduced conservational efforts on ecology including eradicating introduced species as such as the American bullfrog population destroying the native biota of many countries

worldwide (Ficetola et al. 2007).

Due to recent anthropogenic events, native biodiversity built over eons of history are under threat and one of the major sources is introduced species (Seehausen et al. 1997). The American bullfrog has been enlisted as the alien invasive species and as this species thrive on the native biota, may bring disaster to the ecology worldwide (Ficetola et al. 2007). It is thus crucial to develop an eco-friendly trap to reduce and halt the expansions of the American bullfrog (Snow et al. 2011).

Chemical pollution released from factories and cars are the not the only problem that destroy our natural habitat but also introduced species as such as the American bullfrog (Vitt et al. 2013). The alien species that were introduced for various reasons as such as to raise income of the local farmers in the case of American bullfrogs or to reduce the population of local pests in the case of cane toads in Australia have resulted with a massive dent on the native biodiversity. Pollution may be an underlying factor that converted the native macrofauna and macroflora of the natural environment, these introduced species have devastating effect on the natural food chain and may influence microflora that may impact native species on a molecular level (Relyea 2001). As the organisms change, so does the macrofauna and macroflora (Fong et al. 2015). The resulting habitat loss and modification along with the global warming were blamed as the major factors in the decline of abundant and uncommon species everywhere (Fong et al. 2016). The population of alien invasive species American bullfrogs should be controlled and reduced as part of the global efforts of conservation science (Snow et al. 2011).

1.3. American Bullfrog Invasion on a Global Scale

Globally, American bullfrogs are listed as alien invasive species, AIS, but simply known as bullfrog in Canada and the United States (Ficetola et al. 2007). American bullfrogs are studied to consume over 61 species of native amphibians and insects inhabiting Korea (National Environmental Science Institute 2014). There were past efforts from the Korean government to halt this abrasive ecological harm on the Korean native amphibian and reptile population to issue traps and hold hunting competitions however traps caught other species and hunting competitions were not enough to reduce the expanding populations of the American bullfrog. The environmental niche and the native biota were negatively affected by their introduction. This research will analyse the two variables genetic-relatedness and familiarity in the mode of mechanism that may be important to form kin recognition in American bullfrog tadpoles to determine whether they discriminate sibling from non-sibling and whether this is influenced by familiarity and by modeling their interaction, this research will be able to provide basis to the development of an eco-friendly method to halt the expanding population of non-native American bullfrog thereby reducing their detrimental impact on the native amphibian species and simultaneously without polluting the environment.

Almost half of the major historic extinctions arise from introduced animals. The adverse effects caused by introduced species are responsible for the reduction in the richness of local biodiversity and drive native species to extinction. The Nile perch that was introduced to Lake Victoria in early 1980 drove over two hundred species of cichlid fish to endemic extinction

between 1984 and 1997 (Seehausen et al. 1997). Another historic extinction case in Australia involves introduction of the non-native predators as such as the Red Fox that caused almost half of the mammalian species to go extinct in the past 200 years. (Smith et al. 1994, Kinneer et al. 1998) Other factors like disturbance events that lead to habitat clearing and introduced herbivores as competitors were lesser cause (McKenzie et al. 1989, Krebs et al 2009). Cane toads were first introduced to Australia from Hawaii in 1935 to control the native grey-backed cane beetle and French's beetle. However this resulted to the rapid spread of the cane toads to the extent they have become local pests. With the attention of the social media, the scientific efforts to reduce the population of cane toads were then excised and part of that effort was the development of eco-friendly traps to lure only cane toads at tadpole stage at streams and rivers (Zielinski 2012).

As well demonstrated by the three above examples, introduced alien species is one of the most grave conservation problems today. Many cases of unintended or deliberate introductions of non-native organisms are increasing as global trade industry augmented, with little regard for their conservation consequences (Ruesink et al. 1995). There are many examples of introduced species causing conservation problems today including the American bullfrog and its consumption of nearly 61 species of living native creatures in Korea (National Environmental Science Institute 2014).

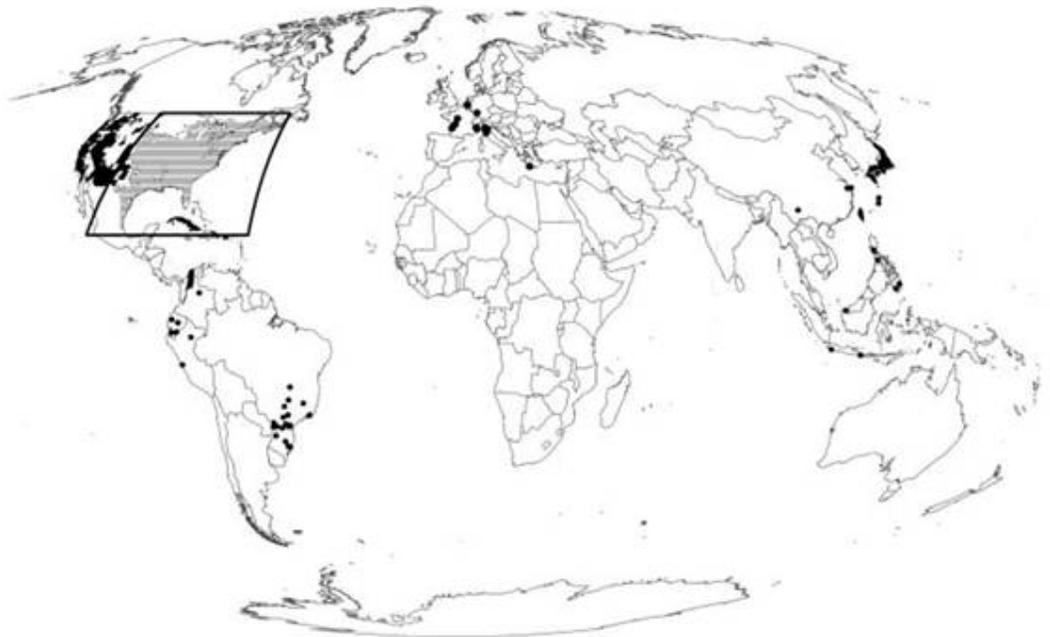


Fig. 1 Map of the distribution of native and non-native populations of *Rana catesbeiana* on a global scale. The barred area represents native populations and black area represents non-native populations. (Santos-Barrera et al. 2004, Ficetola et al 2007).

Since the 1970s, American bullfrogs were imported into South Korea from the United States and Japan to raise the income of local farmers because their hind legs were valued for human consumption (Kim 2009). The American bullfrogs are estimated to have been first released from their import origin in Chonnam province and their local population increased exponentially which devastated native ecology (Kim 2009). The American bullfrogs in the Korean wild led to rapid increase of American bullfrog population in the Korean peninsula due to lack of natural enemies

(Kim 2009). This was a big social issue and the Korean government promoted a hunting competition. The exponential increase of the American bullfrog population size naturally decreased due to presence of racoons, herons and other natural enemies, however they remain as one of the invasive foreign species that are known to consume over 61 various native species (National Environmental Science Institute 2014) and identified as major threat that decrease local biodiversity (Kim 2009, National Environmental Science Institute 2014). Thus thorough research on their kin recognition might provide us with clues to develop eco-friendly traps to control their population without polluting the environment (Snow et al. 2011).

Revealing modes of mechanism by which larval anurans recognize and preferentially relate with their siblings was a substantial task sequel to a discovery of kin recognition amongst them. The conventional studies involved differentiation in ontogeny, ontogeny based plasticity, identification limited to particular settings, and social conditions where sociality was treated as an independent variable (Waldman 1984, Cornell et al. 1989, Fishwild et al. 1990). The American bullfrog, now named *Rana catesbeiana*, was originally named by Mark Catesby (1679/83-1749) as *Lithobates catesbeianus*. They are large light olive, dark green, or brown in color and are heavy-bodied amphibians that have a large tympanum and absent of a dorsolateral fold. Juveniles are pigmented similarly to adults, normally with an olive green color dorsally with many small black spots. These spots may be retained as adults (as brown instead of black), although most juveniles lose the spots as they grow (Dodd 2013). Like the adults, the dorsum of

tadpoles may range from deep olive to light green. *Rana catesbeiana* larvae are the second largest anuran larvae in North America next to River Frog larvae. However, maximum size varies considerably through the ontogeny and may be larval period dependent. *Rana catesbeiana* larvae also are known to display a certain degree of phenotypic plasticity as such as the microbiome on their epidermal layer in response to the presence of predators. A study showed that *R. catesbeiana* tadpoles developed a longer tail fins in the presence of their predators that are mud-minnows (*Umbra*) and *Ambystoma* salamander larvae as relative to their absence (Relyea 2001).

American bullfrogs are native to eastern North America; however, as a result of human introductions, have established populations throughout the southern United States, Hawaii, western Canadian provinces, eight countries of Europe including Belgium, France, Italy, Greece (Ferri et al. 1997, Ficetola et al. 2007), and Korea (Kim 2009). *R. catesbeiana* is one of the most commonly exploited amphibians in the world for over a century. Not only are they frequently used in medical research and laboratory experiments, but also their prime commercial use has been frog legs for human consumption (Dodd 2013). The global trades that resulted in devastating introduction of these frogs into the wild has been originated from implementation of frog farms in both the United States (Arkansas, Louisiana, Florida) and in numerous other countries including South Korea.

In this study we aim to investigate the effect of genetic relatedness and familiarity in *Rana catesbeiana* tadpole that may be important to kin recognition. Our ultimate aim is to enhance our knowledge on the American

bullfrog that is alien invasive species threatening the local biodiversity and find clues to protect our local environment.

1.4. Research objectives

The potential for recognition based on relatedness and familiarity between amphibians above all other vertebrates has been thoroughly researched (Waldman 1991). The mechanism of *Rana catesbeiana* to recognize and identify their kin from non-kin and preferentially associate with kin to non-kin, despite their familiarity, will prove that pre-metamorphose larvae of *Rana catesbeiana* confer benefit through assessment and association behavior.

Conventionally, tadpole's aggregative behavior has been known to serve an aposematic function to the tadpole as it is important to the feeding efficiency, thermoregulation and predator deterrence for the tadpole (Richmond 1947, Brattstrom 1962, Bragg 1965, Waldman et al. 1979). Unlike the schooling behavior displayed by tadpoles, pre-metamorphorsic *Rana catesbeiana* are known to be dispersed without particular schooling. Through this research, I investigated kin recognition in *Rana catesbeiana* tadpoles.

Through this research, our objective is to clarify whether *Rana catesbeiana* kin recognize each other and whether they form social aggregations with their conspecifics based on genetic relatedness and familiarity achieved rearing conditions. Our ultimate aim is to clarify our knowledge on the mode of mechanism that leads to kin recognition in *Rana catesbeiana* tadpoles and in doing so; we would also like to set a basis on

the development of eco-friendly method to facilitate their population without harming our environment. The American bullfrog influence on the local biodiversity is devastating and to establish efficient management methods to facilitate their expanding populations and introductions, insights into their sociality while they are still in their larval stage is imperative.

As American bullfrog initiated ecological damage is detrimental to the Korean biodiversity (Kim 2009), the study aims to test the effect of familiarity and effect of genetic relatedness to *Rana catesbeiana* tadpole behaviour that do not show schooling tendencies during larval stage instead form occasional aggregations in nature (de Avelar et al. 2008). This study aims to apply this information to aid conservation science in efforts to control the invasive non-native species American bullfrog, *Rana catesbeiana* population as such as in the development of eco-friendly method to reduce and/or halt their population growth rate (Snow et al. 2011).

2. Materials and Methods

2.1. Tadpole Rearing Regimes and Testing

The egg masses were collected from Mink Pond located at Estrabrook Woods, Concord Massachusetts in July 1990. The rearing tank was aerated with an airstone, and approximately two-thirds of the tank was substituted with filtered dechlorinated water every two weeks.

2.2. Experimental design

Prior hatching, egg masses were assigned to one of the following two experimental rearing regimes in which two variables, familiarity and genetic relatedness, were manipulated. (1) Control group were composed of familiar siblings: One sibship was reared together in a common tank. (2) Mixed groups were composed of familiar non-siblings: sibships reared together in a common tank were tested as mixed groups. (3) Pure groups were composed of unfamiliar non-siblings: sibships reared separately were placed in the same cylindrical bowl at experiment testing. There were total of 52 pairs that were analyzed. The pairs were analyzed directly from egg mass collected from Mink Pond. Each clutch of eggs was transferred to a separate aquarium, where tadpoles were reared on boiled spinach for about 21 days, until they reached developmental stage 29 (Gosner 1960). Tadpoles were then dyed for 24 hours in 0.0025% aqueous solutions of either methylene blue or neutral red. This staining technique resulted with minimal mortality and marked the tadpoles for several weeks, allowing accurate identification of sibling groups by visual inspection. (Waldman et al. 1979)

For testing total of 8 stained tadpoles released at randomized positions in a 10 cm diameter glass cylindrical bowl depending on the treatment design. Four tadpoles from same clutch were marked the same color of either red or blue. The positions of all tadpoles in the cylindrical bowl were recorded for approximately 20 min. There were 15 trials to control group, 23 trials for treatment pure groups and 14 trials to treatment mixed group.

2.3. Data Collection and Analysis

The recorded tapes were then digitalized for analysis. A computer program for ethological analyses (Observer Version 5.0, Noldus, Wageningen, The Netherlands) was used. For analysis, the frequencies of even behavior, 'Contact' was recorded. Based on Walls (1990) analysis of behavior of juvenile salamanders, for a tadpole to approach a conspecific and making contact (i.e. nibbling on its head) was thought to represent agonistic and assessment behavior in post-metamorphic *Ambystoma* (Walls 1990). A study by de Avelar Sao Pedro (2008) made field observations that tadpoles of *Rana catesbeiana* form social and spherical aggregations where each individuals would either remain in repose or stay next to a substrate. To quantify the naturally formed aggregations, contact was counted as social factor. Contact between two individuals seemed passively agonistic but sometimes less territorial seemed amicable (i.e. close spacing positioned parallel). The behavior itself could have been viewed also as a submissive behavior as the subjected tadpole had to endure 'nibbling' or 'closing in.' 'Contact' represents a passive mechanism by which each individual tadpole

forms aggregation, hence all type of contact interaction between them was counted. Each contact was counted when it was longer duration than approximately 1 second as sometimes it was a passive aggression of tapping and swim away. The behavior 'contact' was counted between same colors, red-red, blue-blue, and other colors, red-blue, blue-red.

Statistical methods

To test the hypothesis that tadpoles discriminate and preferentially associate with kin as opposed to non-kin, the data profiles containing statistical information on experimental behavior acquired from the Observer 5 were analysed. The exported data from Observer 5 contained information on sum of activity and the rate of activity, where activity in this case is 'contact.' The sum of contact count between each colored tadpoles to their same colored conspecific and different colored conspecifics were then calculated. Rate of contact is number of contact by each colored tadpoles either to same or different colored conspecifics made per minute. I initially calculated mean values of red and blue making contact to same color (kin) and to other color (non-kin) for each trials of treatment groups. To statistically pairwise determine the effect of the two variables 1) genetic relatedness 2) familiarity between each group by their activity count I conducted two-tailed ANOVA tests using Minitab 18. To consider the significance of 'genetic relatedness', I analysed the differences in variation between the two following experimental conditions 1) familiar-sibling: control group and 2) familiar-non-sibling: mixed group. To check the significance of

‘familiarity’ as a variable, I analysed the differences in variation of contact frequency between two following experimental conditions 1) familiar-non-sibling: mixed group 2) unfamiliar-non-sibling: pure group. I also conducted Wilcoxon signed-rank tests to evaluate the levels of significance (Non-Parametric, two-tailed) for statistical comparisons of aggregation pattern for each treatment groups and by the color of tadpoles between the same colored conspecifics and different colored conspecifics to each treatment group.

3. Result

3.1. Effect of Genetic Relatedness

The result of the analysis showed that there was no difference to levels of contact between juvenile *Rana catesbeiana* tadpoles and they displayed not significantly different levels of contact to siblings than to non-siblings. Data showed that both in the familiar non-sibling groups and the unfamiliar sibling groups, the contact count of assessment of siblings to their non-siblings was higher by 1.321 counts in the familiar non-sibling group and 1.281 in unfamiliar non-siblings. Moreover, the juvenile bullfrog tadpoles exhibited higher levels of contact rate to their siblings than to non-siblings. Lower levels of contact rate between juvenile tadpoles toward familiar non-siblings were shown in the mixed group. Figure 2 shows that the levels of contact were at higher levels amongst tadpoles of the same color then of that towards tadpoles of the other color, considering the probability rule where the tadpole would have higher chance of associating with the tadpoles of the other color than of the same color because the tadpole of the same color is one less (subtracting the subjected-tadpole), 3, than to other color which is 4 to a total of 8 tadpoles. The probability rule is reflected in the control group where the mean activity count of control is about 23.4% (Expected value 25%: Observed value 23.38%) higher to associate with other color coded tadpole then that of the same color code. The level of contact overall intensified to both the familiar and unfamiliar non-kin in the pure group, possibly due to tadpoles acclimatizing and assessing whether the neighbour tadpole is possible prey item or a kin.

Result of analysis of variance on genetic relatedness further substantiated that genetic relatedness in tadpoles does not make significant difference to *Rana catesbeiana* tadpole spherical social occasional aggregation.

3.2. Effect of Familiarity

Spatial knowledge acquired during juvenile dispersal or tadpole mediates homing behavior in the neotropical territorial frog, *Allobates femoralis* (Pašukonis et al. 2014). It was predicted that tadpoles predisposed to their genetically related siblings and non-related but familiar siblings and would show differences in the level of aggregation formation at experiment testing. Unfamiliar and not genetically related tadpoles was hypothesized to show definite differences in the level of aggregation formation. However, the bullfrog tadpoles observed in this experiment were three-weeks old and to claim the possibility of territoriality of tadpoles, still at their infancy is rather perplexing. Rather, the knowledge of the surrounding environment learnt at the tadpole stage may aid them at adult frog stage as such is the case in poison dart frogs returning back to their hatching spot (Pašukonis et al. 2014). Familiar *Rana catesbeiana* tadpoles did not show significant difference to discriminate between kin and non-kin. The familiar *Rana catesbeiana* tadpoles in the mixed group showed same levels of contact to their familiar non-kin. The familiar *Rana catesbeiana* tadpoles in the pure group showed same levels of contact to their unfamiliar non-kin. The results of analysis of variance showed that familiarity to have no difference in tadpoles to associate with conspecifics (Table 4).

Table 1 Mean of total contact count of all trials and the standard deviations

	Same Color	Other Color
Mean Contact Count of Control	18.64	24.33
Mean Contact Count of Pure	24.52	23.81
Mean Contact Count of Mixed	23.39	22.07
Standard Deviation of Activity Count of Control	1.85	5.34
Standard Deviation of Activity Count of Pure Group	1.52	4.09
Standard Deviation of Activity Count of Mixed Group	0.25	3.934

In the experimental tests, egg clutches were assigned to tanks following 3 experimental regimes in which two variables (1) genetic relatedness and (2) familiarity, were manipulated. There were three experimental conditions each named “Control”, “Pure” and “Mixed” group. The 3 groups were as follows (1) control group were composed of familiar siblings: Egg mass were reared together in a common tank, (2) pure group was composed of unfamiliar non-siblings: four tadpoles, each from separate tanks, were dyed different colors and released at randomized positions at a 10x10 cm cylindrical bowl for experimental observation. The (3) mixed group was composed of familiar non-siblings: egg masses of different sib-ship were reared together in a common tank. The tadpoles of different sibships were stained different colors for identification. The mean contact count for each group, of tadpoles making contact to the tadpole of same color and other color were calculated. The mean contact count of tadpoles making contact towards same color was highest in pure groups. The mean activity count of tadpoles making contact towards other color was highest in the mixed groups.

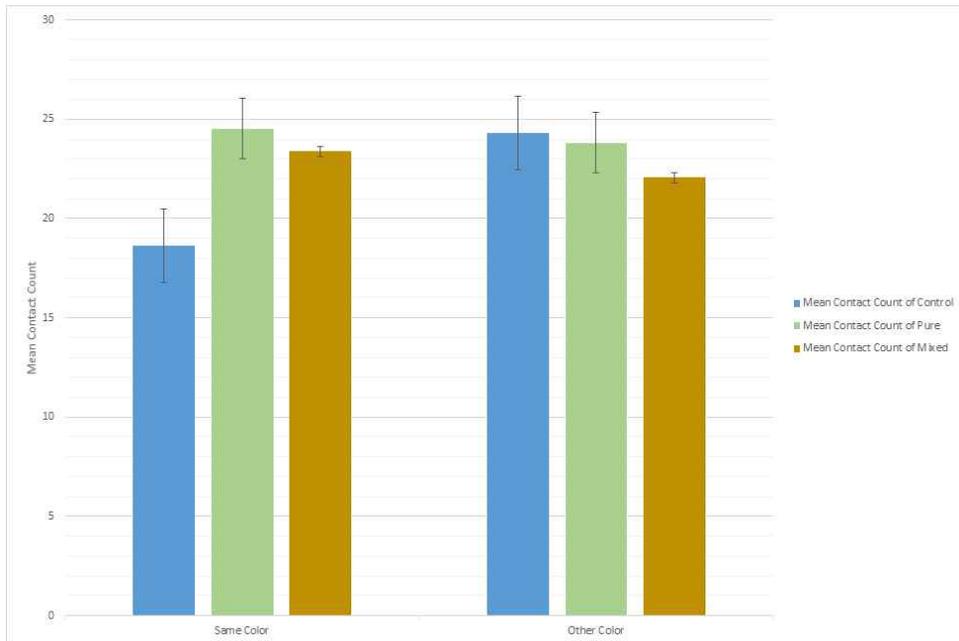


Fig. 2 Mean contact count between 3 different experimental groups. The bar graph shows that there is highest level of tadpole contact in pure group to a mixed group. The levels of contact of the tadpole making assessment towards same color coded tadpole is relatively higher in both the pure and mixed group. The error bars indicates standard deviations which is a measure of dispersion of a set of data values. The contact count of control group is composed of tadpoles of same sibship reared together in a common tank. The difference in the variation of contact count of control group from same color to other color reflects the probability rule where the mean activity count of control is about 23.4% (Expected value 25%: Observed value 23.38%) higher to associate with other color coded tadpole then that of the same color code.

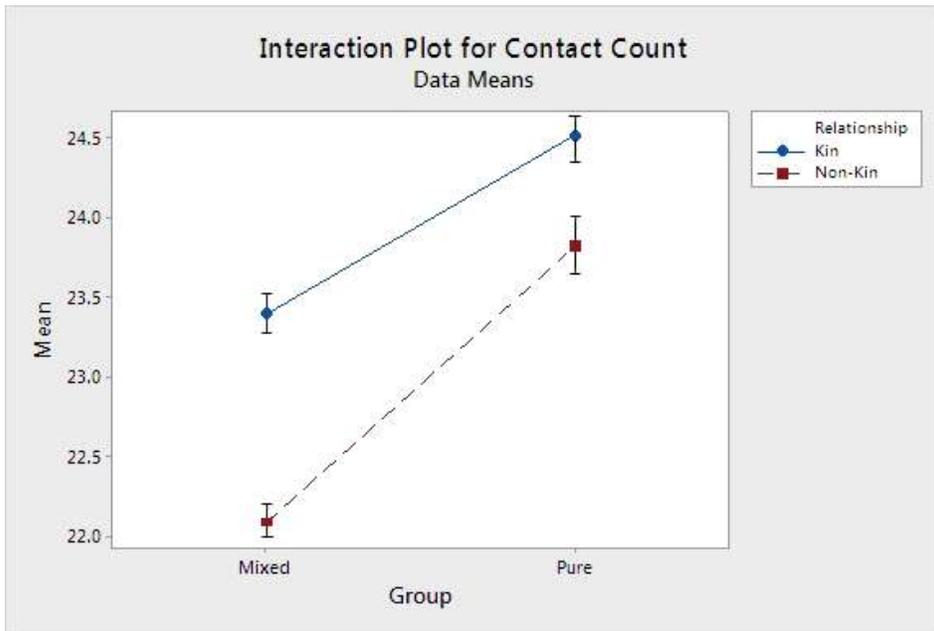


Fig. 3 Interaction plot of contact count between tadpoles of mixed and pure group. Tadpoles of pure group showed elevated level of contact compared to those of mixed group.

Table 2 Analysis of Variance for Experimental Tests

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group (Control, Mixed, Pure)	2	76.1	38.06	0.13	0.879
Familiarity (Other, Same)	1	97.9	97.94	0.33	0.565
Group*Familiarity Interaction	2	554.2	277.11	0.94	0.392
Error	218	64223.0	294.60		
Total	223	64907.4			

Three sources of variation and their interactions were considered. Group refers to the experimental conditions and familiarity refers to the tadpoles

assessing conspecifics of other color coded tadpole and of same color coded tadpole. Group and familiarity interactions were considered.

Table 3 Analysis of Variance of Control and Mixed group

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group (Control, Mixed)	1	30.0	30.01	0.10	0.750
Familiarity (Other, Same)	1	188.8	188.83	0.64	0.424
Error	113	33123.4	293.13		
Lack-of-Fit	1	406.0	406.04	1.39	0.241
Pure Error	112	32717.4	292.12		
Total	115	33342.2			

The differential values between the variances of control (familiar siblings) and mixed group (familiar non-siblings) show the effect of the conditioned variable which in this case is ‘ontogeny.’

Table 4 Analysis of Variance of Mixed and Pure group

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group (Mixed, Pure)	1	75.9	75.890	0.31	0.579
Familiarity (Other, Same)	1	34.3	34.299	0.14	0.709
Error	161	39559.7	245.712		
Lack-of-Fit	1	3.5	3.518	0.01	0.905
Pure Error	160	39556.2	247.226		
Total	163	39669.9			

The differential values between the variances of mixed (familiar non-siblings) and pure group (unfamiliar non-siblings) show the effect of the conditioned variable which in this case is ‘familiarity.’

Table 5 Analysis of Variance of Control and Pure group

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group (Control, Pure)	1	6.7	6.696	0.02	0.889
Familiarity (Other, Same)	1	128.6	128.625	0.37	0.541
Error	165	56627.6	343.198		
Lack-of-Fit	1	455.2	455.162	1.33	0.251
Pure Error	164	56172.5	342.515		
Total	167	56762.9			

The differential values between variances of control and pure group were considered. Although two variables did not overlap, a value of 0.37 was represented probably because the choices that each tadpoles of control group made to same or other color coded conspecific was not affected by either ontogeny or familiarity.

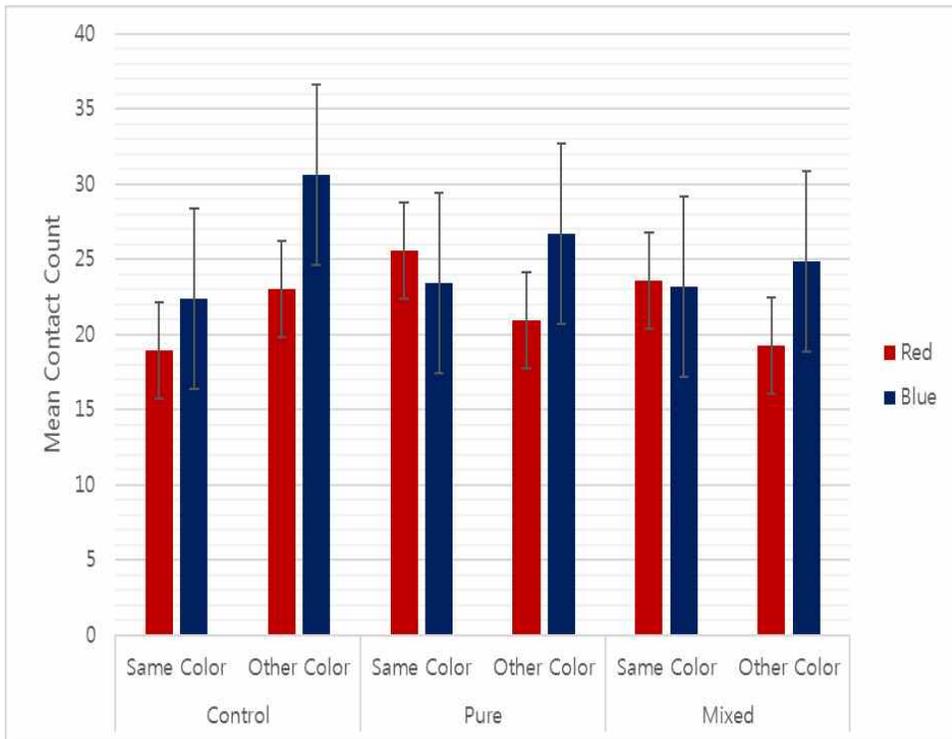


Fig. 4 Mean contact count of red and blue tadpoles of 3 different treatment groups. Mean contact count of red tadpole and blue tadpoles due their experimental conditions represented. The mean contact levels of the pure group is higher than that of the mixed group. In both groups, the tadpoles making contact to those coded the same color were higher to those coded other color.

Table 6 Levels of significance (Non-Parametric, two-tailed) for statistical comparisons (Wilcoxon signed-rank tests) of passively agonistic and assessment behavior between each treatment groups

Comparison	N for Test	Wilcoxon Statistic	P-value	Median
Control group: Contact between same colored sibling and other colored familiar-sibling	28	100	0.02	-5
Mixed group: Contact between same colored sibling and other colored familiar-non-sibling	24	175	0.484	1
Pure group: Contact between same colored sibling and other colored unfamiliar-non-sibling	49	681.5	0.496	0.5

The values of N and Wilcoxon statistic and P-values are represented. Control group demonstrates significance in the aggregation between same colored tadpoles to other colored tadpoles then to other treatment groups. The P-value(=0.02) shows that the data set of control group is highly significant. This is due to probability rule where the tadpole would have higher chance of associating with the tadpoles of the other color than those of the same color because the tadpole of the same color is one less (subtracting the subjected-tadpole), 3, than to other color which is 4 to a total of 8 tadpoles. The probability rule is reflected in the control group where the mean contact count of control is about 23.4% (Expected value

25%: Observed value 23.38%) higher to associate with other color coded tadpole than that of the same color code.

Table 7 Levels of significance (NP, two-tailed) for statistical comparisons (Wilcoxon signed-rank tests) of passively agonistic and assessment behavior for Red and Blue colored tadpoles of each treatment groups

Comparison	N for Test	Wilcoxon Statistic	P-value	Median
Control group: Red tadpoles Contact to same colored familiar-sibling and other colored familiar-sibling	14	28	0.132	-4.5
Control group: Blue tadpoles Contact to same colored familiar-sibling and other colored familiar-sibling	14	24	0.079	-7.5
Mixed group: Red tadpoles Contact to same colored familiar-sibling and other colored familiar-non-sibling	14	77.5	0.124	3.5
Mixed group: Blue tadpoles Contact to same colored familiar-sibling and other colored familiar-non-sibling	10	20	0.476	-0.5
Pure group: Red tadpoles Contact to same colored familiar-sibling and other colored unfamiliar-non-sibling	23	213	0.023	2
Pure group: Blue tadpoles Contact to same colored familiar-sibling and other colored unfamiliar-non-sibling	26	119	0.155	-2

The table shows the results of Wilcoxon's tests for consideration.

4. Discussion

4.1. Sociality of *Rana catesbeiana* tadpoles

Rana catesbeiana tadpoles are known to form occasional aggregations in the wild (de Avelar et al. 2008). The results showed that the two variables 1) genetic relatedness and 2) familiarity did not make difference for *Rana catesbeiana* tadpoles to make contact.

The data analysis showed no significance (P-values>0.05) therefore, there was no difference between the two variables genetic-relatedness and familiarity in the mechanism of kin recognition in *Rana catesbeiana* tadpoles. Because both their level of contact count and the rate of contact were relatively low towards familiar individuals that were not related to them by gene, the low levels probably accounts to the fact that they have already completed assessment to know that they are non-kin and not particular good food source either so displayed the least interest. The low levels activity in control group implies that *Rana catesbeiana* would not particularly socialize and interact with their kin since their assessment has ended. This agrees with their ecological natural state in which they remain independent and form occasional social aggregations in the pond without particular schooling (de Avelar et al. 2008).

Tadpoles of *Rana catesbeiana* manifest occasional social and spherical aggregations in nature (de Avelar et al. 2008) The result of the study indicates support between direct relative support (as close as sibling) rather than species support. The result of the analysis of the data obtained from the recorded tapes of the eight tadpoles enclosed together in a 10 cm

diameter bowl with 1) familiar sibling 2) familiar non-siblings 3) unfamiliar non-siblings, revealed that there was no significance difference between them. Since ‘contact’ behavior could be amicable behaviour of assessment (Hamilton et al. 1967, Wrobel et al. 1980, Walls 1990), this could be the explanation to the increase of contact behavior towards their unfamiliar non-sibling.

Ever since the discovery, the ability of amphibian tadpoles to recognize and school with their siblings had been thoroughly researched (Waldman 1984, O’Hara et al. 1985, Cornell et al. 1989, de Avelar et al. 2008). The tadpoles to aggregative behaviour may have conferred benefit to not only the individual itself but also increased inclusive fitness of the same sibship and close relatives – group selection. As opposed to being alone, being in a group serves the tadpole with increased feeding efficiency, thermoregulation and predator deterrence (Richmond 1947, Brattstrom 1962, Bragg 1965, Waldman et al. 1979). The 21 day old tadpoles of *Rana catesbeiana* have shown no significance difference to the two variables genetic relatedness and familiarity to make contact with kin. Although tadpoles of *Rana catesbeiana* do not school in the natural environment, as a result of a genetic effect, would prefer to associate with kin mainly for the purposes of feeding efficiency and deterrence from predators because genes are shared between the siblings of same sibship. The interaction plot for rate of activity further substantiates the result. The interaction between juvenile tadpoles of *Rana catesbeiana* is consistent between the mixed and pure groups. Although the level of interaction increased slightly as tadpoles were exposed to unfamiliar conspecific, the increased levels were consistent

with the tadpoles' association towards non-kin. The interaction plot for tadpoles to associate with their non-siblings when they are familiar is relatively low compared to their interaction rate when they are unfamiliar non-siblings. This abrupt increase in the tadpoles to assess unfamiliar non-kin indicates that the tadpoles could have also assessed the members of the other sibship with the possibility to prey upon them (Waldman et al. 2015). The allele for this behaviour could have evolutionarily favoured for its enhanced hunting abilities that would confer benefit to the individual's survival. There was no significance between the genetic relatedness and familiarity for *Rana catesbeiana* tadpoles to contact each other.

4.2. Implications of familiarity

The result of differences in variation obtained through analysis of variance tests provides an experimental data that *Rana catesbeiana* base their social interaction with no differences in genetic relatedness and familiarity. Comparison on the obtained statistical data showed that the tadpoles of *Rana catesbeiana* form occasional social contact with no significant difference between the two variables, genetic relatedness and familiarity. Lack of significance on the data analysis indicated that further research must be excised to determine kin recognition in *Rana catesbeiana*. The two variables 1) genetic relatedness and 2) familiarity have been manipulated in this experiment to find the impeding factor for *Rana catesbeiana* social interaction modality. The tadpoles illustrated lowest mean contact count towards a non-genetically related, but familiar individual. This might be that

tadpoles 'learnt' they are non-sibling and not a prey item during their rearing period. The allele for this trait could have been selected for because they would have to compete for food in the ecological niche without providing particular predator deterrence and feeding efficiency from sharing ontogenetic cues. The juvenile tadpoles of *Rana catesbeiana* showing elevated levels of contact to their unfamiliar conspecific could have resulted from assessing whether they could prey on them or halt predation from a possible predator (Waldman and Adler 1979, Wrobel et al. 1980, Garza and Waldman 2015). Familiar individuals would share indirect cues to various signals obtained from the environment. These might be contingent on the circumstances in which they encounter (Waldman 1991).

5. Conclusion

This study provides appreciable experimental data to be considered for *Rana catesbeiana* tadpoles to form occasional social ‘contact’ that does not show differences in the two variables ‘genetic-relatedness’ and ‘familiarity.’ To determine, with high significance, the degrees of genetic relatedness with variation in degree of familiarity amongst tadpoles of *Rana catesbeiana* that may form kin recognition, further study is impetus. The analysis on the data showed no significance in the differences in the two variables, genetic relatedness and familiarity except the control group which might be due to the fact that the treatment groups were both affected by the probability rule. Lack of significance of the treatment groups in the data analysis could be also to the fact that since contact was assumed to be a quantifiable indicator of occasional aggregations that occur in the wild, the procedure to count contact involved each subjected tadpoles making effort to make contact. For example, when there is a contact made which was an approach from one tadpole it would be counted once but when there is a contact made by approaches made from two tadpoles then the contact would be counted twice, thus ‘effort’ by each tadpole was unintentionally measured as a separate variable to count contact by using Observer 5. Therefore the lack of significance could be because every contact were counted once(single tadpole approach to make contact) or twice (two tadpoles approach each other to make contact) depending on the number of tadpole making an effort to approach. *Rana catesbeiana* tadpoles do not school in the wild, and their high levels of assessment towards their unfamiliar-non-kin showed that this assessment behaviour was selected for them to deter predation and

effectively assess for possible prey item. Their lack of hesitation to approach and assess on their unfamiliar-non-kin contributes as one of the key qualities that determine them to be one of the top predators in ecological niche and pose threat to the native amphibian populations on a global scale (Fig 1). The result that there was no difference with the two variables, genetic-relatedness and familiarity to the *Rana catesbeiana* tadpoles to make contact could be the result from their infancy of 21 days post hatching when their larval stage last for 2 years and possibly from using a computer program for ethological analyses 'Observer 5' whereby 'effort' was scored unintentionally as another variable. Further study could be done to determine kin recognition in *Rana catesbeiana* not only at 21 days post hatching but also at 2 year larval stage to see whether this preference to kin association could be due to the inherited trait which protect them at their infancy when they still are very small and vulnerable to predation and at 2 years age, they would behave differently due to their large body size that go up to 5 centimetres as a larva. This would aid us in understanding temporal mechanisms to kin recognition of *Rana catesbeiana* in their life cycle.

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Appendix

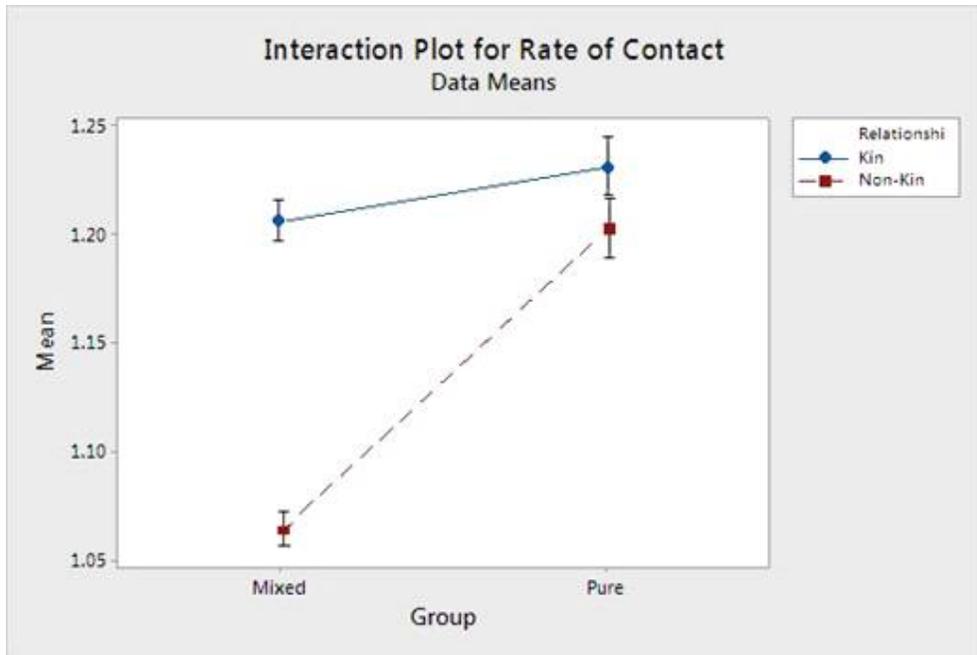


Fig. A1 Interaction plot for rate of contact count made per minute between mixed familiar non-sibling and pure unfamiliar non-sibling groups. This interaction plot for rate of contact count made per minute for each tadpole in the groups substantiate the finding that these tadpoles would make assessment towards their unfamiliar non-kin without hesitation possibly to identify whether their unfamiliar conspecific is genetically related to them and whether they make a good prey item (Waldman et al. 2015). As *Rana catesbeiana* are successful as one of the apex amphibian predators in the food chain in the natural ecological niche, and enlisted as Alien Invasive Species, the allele for this behaviour might have been selected for, and may be accentuated later in developmental stage. The *Rana catesbeiana* tadpoles in this experiment were 21 days old.

Table A1 Analysis of Variance of three groups and their interactions were considered. The data was obtained from Observer 5 Noldus in Activity Rate Number Per Minute format.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group (Control, Mixed, Pure)	2	0.298	0.14893	0.20	0.818
Familiarity (Other, Same)	1	0.089	0.08927	0.12	0.728
Group*Familiarity	2	1.596	0.79785	1.08	0.342
Error	206	152.200	0.73884		
Total	211	154.145			

Table A2 Analysis of Variance of control and mixed groups were considered. The data was obtained from Observer 5 Noldus in Activity Rate Number Per Minute format.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group (Control, Mixed)	1	0.0066	0.00660	0.01	0.924
Familiarity (Other, Same)	1	0.1786	0.17864	0.25	0.619
Error	117	84.1147	0.71893		
Lack-of-Fit	1	1.4498	1.44980	2.03	0.156
Pure Error	116	82.6649	0.71263		
Total	119	84.3000			

Table A3 Analysis of Variance of mixed and pure groups were considered. The data was obtained from Observer 5 Noldus in Activity Rate Number Per Minute format.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group (Mixed, Pure)	1	0.2454	0.2454	0.39	0.532
Familiarity (Other, Same)	1	0.2063	0.2063	0.33	0.567
Error	149	93.2227	0.6257		
Lack-of-Fit	1	0.1179	0.1179	0.19	0.666
Pure Error	148	93.1048	0.6291		
Total	151	93.6744			

Table A4 Analysis of Variance of control and pure groups were considered. The data was obtained from Observer 5 Noldus in Activity Rate Number Per Minute format.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group (Control, Pure)	1	0.165	0.1648	0.19	0.664
Familiarity (Other, Same)	1	0.379	0.3790	0.44	0.510
Error	149	129.594	0.8698		
Lack-of-Fit	1	0.963	0.9631	1.11	0.294
Pure Error	148	128.631	0.8691		
Total	151	130.138			

국문초록 (Abstract in Korean)

혈연 인식(Kin recognition)은 동물이 서로 다른 각 개체와 혈연도의 원근을 식별하여 그에 따라 행동을 바꾸는 것이라고 한다. W.D. Hamilton의 혈연선택이론에 따르면 동물의 각 개체는 혈연도에 따라 행동을 바꾸는 것이 동물의 생존율에 기여하며 그 때문에 상대와 혈연도를 식별하는 것이 중요하다고 주장하였고, 그에 따른 다양한 연구가 각 응용분야에서 진행되어 왔다. 그러나 생태학적 측면으로는 그 구체적인 기구가 충분히 밝혀진 바가 없다.

이에 본 연구는 행동 분석에 특화된 (Observer 5, Noldus) 프로그램을 이용하여 자연적으로 무리 지어 (schooling) 유영하지 않는 황소개구리 (*Rana catesbeiana*) 올챙이의 혈연 인식 기구를 알아보는 것을 목적으로 수행되었다. 혈연 인식에 기여하는 요소로 크게 유전적 관련성 (genetic relatedness) 과 환경적 친숙성 (familiarity) 으로 분류하여 그에 따른 행동 (contact) 을 상대 평가(assessment) 와 간접적인 투쟁 (passively agonistic)을 대표하는 측정 변수로 기반을 두었다.

각 요소를 다르게 한 그룹에 대한 통계적으로 분석한 결과 유전적 관련성이 환경적 친숙성보다 혈연 인식에 더 크게 작용하는 것으로 밝혀졌다. 비모수 통계 검정 (Wilcoxon signed-rank test) 결과에 의하면 유의성이 다소 낮은 것으로 나타났으나, 생태적으로 황소개구리 올챙이의 분산 양식이 기존의 무리지어 유영 (schooling)하는 일반 무미목 올챙이와 다르기에 유전적 관련도로 혈연 인식하여, 생태적 지위 (ecological niche)에서 보다 획기적으로 장악한다는 가설에 잠재성을 시사한 결과였다. 또한 본 연구 결과는 '새 환경 순응'이 (acclimatization) 혈연 인식

에 상호작용하여 일어 날 수 있음을 보여주었다.

주요어: 혈연 인식, 황소개구리 개체 간의 상호관계, 혈연도, 친숙성,
Rana catesbeiana

학번: 2016-29360

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이 논문이 나오기 까지 많은 분들의 도움이 있었습니다. 가장 먼저 지도 교수이신 Bruce Waldman 박사님께 깊은 감사의 마음을 전하고 싶습니다. 처음 실험실 생활을 시작했을 때가 기억에 생생합니다. 어리고 경험이 미숙했던 저를 숙련된 과학자 선배로서 그리고 삶의 선배로 진심 어린 조언들로 저를 이끌어 주셔서 감사했습니다. 어릴 때부터 외국 생활을 해서 한국 문화나 생활에 문외한이었던 제가 석사 기간 2년간 연구실 생활에 정착하게 이끌어 주신 저의 소중한 실험실원들 한분 한분 머리 숙여 감사드립니다.

흔쾌히 논문심사를 맡아주신 Piotr Jablonski 교수님과 민미숙 교수님께 깊이 감사의 말씀을 전하고 싶습니다. 첫 학위논문이 였던 탓에 미숙한 점들이 있었지만, 교수님들의 조언을 발판으로 많이 발전 하였습니다. 석사 생활 동안 저에게 간간히 진심 어린 조언 해주신 이은주 교수님께 머리 숙여 감사드리며, 바쁜 일정 가운데에도 합심하여 저에게 도움의 손길 주신 석영재 교수님과 정종경 교수님 너무 감사드립니다.

매일같이 동거 동락하였던 연구실 식구들에게 다시 한 번 감사드립니다. 함께 한 시간은 길지 않아도, 어떤 소식에도 미소로 반겨주신 Dharmo 박사님, 만년방장 푸민지에게, 더불어서 연구실에 유일한 다른 한국인 석사생 이셨던 이은선씨는 저의 석사과정 중의 개인적 위기 상황에서 진심 어린 조언으로 제게 큰 위안과 용기를 주셨습니다. 미숙했던 제가 연구실 생활에 적응하게끔 아낌없는 지지와 격려를 베풀어주신 신재협 선배님과 사만다에게 감사드립니다. 그리고 첫 학기에 진행했던 청개구리 실험을 함께했던 우재호 학부생과 이도나 학부생, 성가심의 내색 한 번 없이 분위기를 탐구적이면서도 훈훈하게 이끌어 주어 감사합니다. 같이 몰두꺼

비 실험을 진행했던 인턴 Jesse도 실험 관련 Observer 프로그램에 대한 친절한 조언과 아낌없는 격려 감사합니다. 낯설던 연구실 환경이 차츰 차츰 친화적으로 다가왔던 건 여러 학부생 분들과 인턴 분들 덕분 이였습니다. 프로젝트의 위기상황에 진심어린 조언 아낌없이 해준 전종윤 학부생과 김주완 학부생도 감사합니다. 미숙했던 저의 논문에 사려깊은 리뷰를 해준 Miranda도 감사합니다.

미숙했던 저의 인턴 생활을 진심어린 권유와 조언들로 잡아주신 정구흥 교수님께 머리 숙여 감사드립니다. 저의 바로 뒷자리에 앉아 선배 과학자로서 각종 클로닝 기법에 대한 팁과 쏠쏠한 말동무가 되어주신 서현욱 선배님께도 감사드립니다. 진정으로 과학 연구를 간구하는 모습들로 저에게 큰 감동 되어주신 고은경 박사님께도 깊이 감사드립니다.

머나먼 미국 대학원 생활 속에서도 진심어린 조언과 선배 과학자로서 아낌없이 격려 해준 이우식과 도서관 사서에서 이제 어엿한 삼성 SDS에서 근무하게 된 강준우도 마음 속 깊이 감사드립니다.

여러 어려움 가운데도 저에게 항상 큰 힘과 동기 되어주시고, 세상 유일하며 한분이신 하늘에 계신 그분께도 감사합니다. 글로 다 적어 낼 수 없이 휘황찬란했던 석사생활 동안 아침마다 저에게 버팀목이 되어 주시고, 또 Campus Crusade for Christ 라는 좋은 동아리에 다리를 놓아준 강호찬 학부생과 김유정, 이제는 대학원생, 그리고 우종학 교수님을 만나게 해주셔서 감사하고 또한 제 삶의 풍부한 내적 경험을 비롯해 한국 생활에 무사히 안착 하게 저를 인도 해주어 감사드립니다. 가장 최근에 만났던 예수 하면 떠오르는 우종학 교수님, 선배 과학자로서 올바르게 과학과 종교를 바라보는 관점을 보여주시고 또 이러한 내적으로 풍부한 삶 허락하셔서 마음 속 깊이 감사드립니다.

같이 독거노인 자원봉사도 가고 여러모로 저를 온정 가득히 인도 하여주신 한혜원 순장님 감사드립니다. 집과 연구실만 오가던 제가 뮤지컬도 가고 신나는 시간 허락하신 이현지 순장님을 감사드리며, 같이 지내며 내적으로 풍부하며 포근해지는 시간 허락한 김혜민 순장님과 홍혜림 순장님 마음 깊이 감사드립니다. 화요채플과 금요일예배시간을 거룩하게, 말씀으로 은혜롭게 이끌어주신 최수찬 간사님 또한 감사드립니다. 1년간 미국 아틀란타로 단기 선교를 가시는 이은 순장님, 처음 같이 자원 봉사 갈 때 어색했지만, LTC 강사로 섬기시는 걸 함께하고 감동의 순간을 함께하여 감사합니다. 다 적을 순 없지만 갔을 때 순도 함께하고 식사생활에도 활기찬 종교 생활을 지지하여준 주은 인아 서혜 민지 시은 예형 순원 분들과 다빈 상윤 경환 순장님들 감사드립니다.

저에게 여러모로 아낌없는 지지와 격려 해주신 부모님께도 감사드립니다. 든든한 후원자로 그리고 조연자로 저에게 힘이 되어주신 아버지와 오래된 친구처럼 포근한 조연과 간간히 은혜로운 말씀을 전해주신 어머니 사랑합니다. 이제 갓 결혼한 언니도 형부와 오랜 신혼생활을 영위하길 바라고 식사생활에 대한 조연 아낌없이 해주어 고마워. 그리고 부산 집 들를 때마다 아낌없는 꼬리치기로 반가움을 표현한 시바견 하쿠 사랑해.